



# Digenic inheritance in autosomal recessive non-syndromic hearing loss cases carrying *GJB2* heterozygote mutations: Assessment of *GJB4*, *GJA1*, and *GJC3*

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## ABSTRACT

**Objective:** Autosomal recessive non-syndromic hearing loss (ARNSHL) can be caused by many genes. However, mutations in the *GJB2* gene, which encodes the gap-junction (GJ) protein connexin (Cx) 26, constitute a considerable proportion differing among population. Between 10 and 42 percent of patients with recessive *GJB2* mutations carry only one mutant allele. Mutations in *GJB4*, *GJA1*, and *GJC3* encoding Cx30.3, Cx43, and Cx29, respectively, can lead to HL. Combination of different connexins in heteromeric and heterotypic GJ assemblies is possible. This study aims to determine whether variations in any of the genes *GJB4*, *GJA1* or *GJC3* can be the second mutant allele causing the disease in the digenic mode of inheritance in the studied *GJB2* heterozygous cases.

**Methods:** We examined 34 unrelated *GJB2* heterozygous ARNSHL subjects from different geographic and ethnic areas in Iran, using polymerase chain reaction (PCR) followed by direct DNA sequencing to identify any sequence variations in these genes. Restriction fragment length polymorphism (RFLP) assays were performed on 400 normal hearing individuals.

**Results:** Sequence analysis of *GJB4* showed five heterozygous variations including c.451C>A, c.219C>T, c.507C>G, c.155\_158delTCTG and c.542C>T, with only the latter variation not being detected in any of control samples. There were three heterozygous variations including c.758C>T, c.717G>A and c.3\*dupA in *GJA1* in four cases. We found no variations in *GJC3* gene sequence.

**Conclusion:** Our data suggest that *GJB4* c.542C>T variant and less likely some variations of *GJB4* and *GJA1*, but not possibly *GJC3*, can be assigned to ARNSHL in *GJB2* heterozygous mutation carriers providing clues of the digenic pattern.

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## 1. Introduction

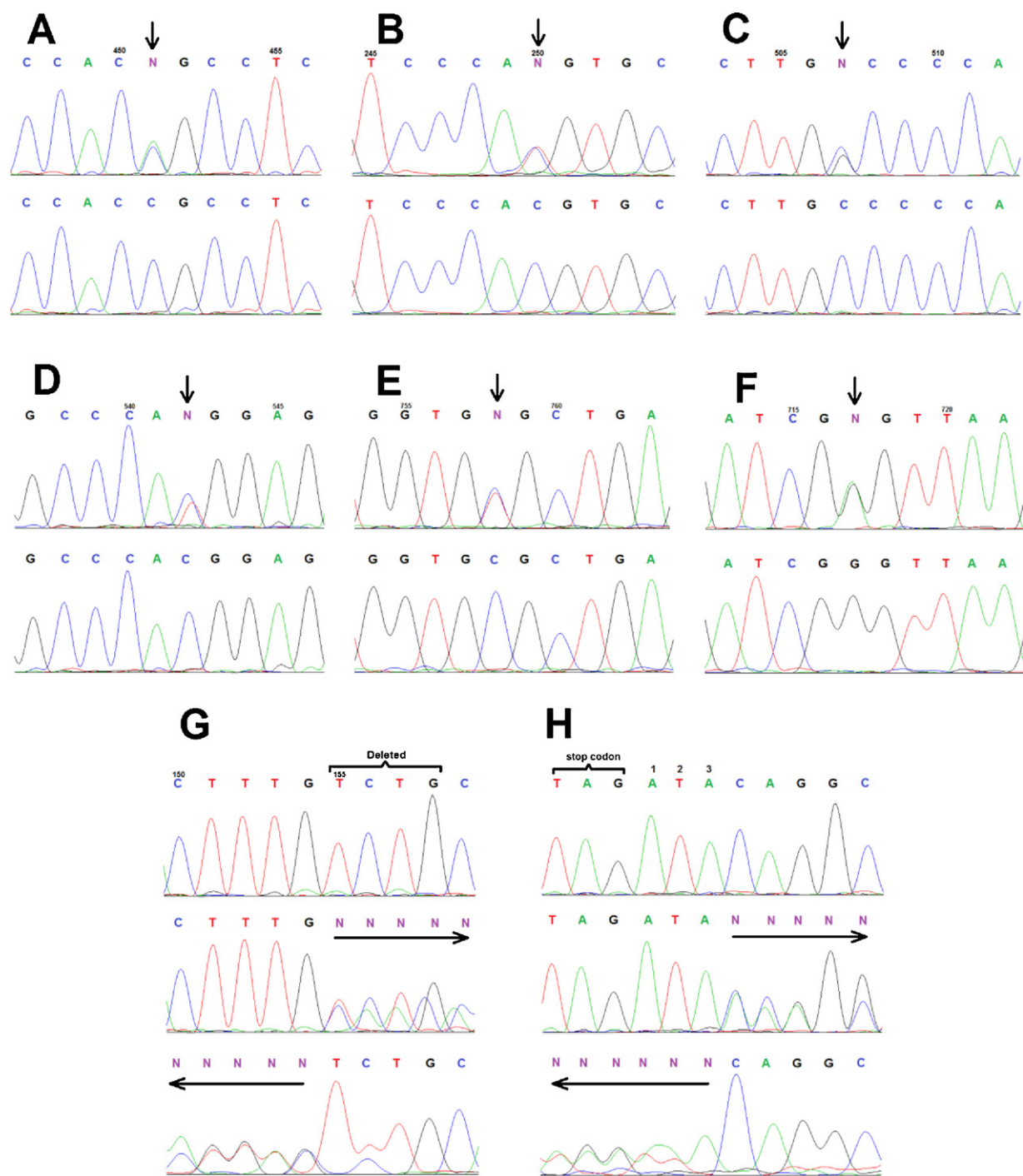
Hearing loss (HL), with an incidence of about 1 in 1000 neonates, is the most frequent sensorineural disorder. It is extremely heterogenous and may occur due to genetic or environmental causes or both [1,2]. Despite this genetic heterogeneity, mutations in the *GJB2* gene (MIM ID: 121011) which encodes connexin (Cx) 26 protein, are involved in up to 50% of autosomal recessive non-syndromic HL (ARNSHL) cases in many populations

[3]. From 10 to 42 percent of patients with *GJB2* mutations are heterozygous carriers of a mutant *GJB2* alleles [4–10]. Cxs are the building blocks of gap junctions (GJs) that build intercellular channels allowing communication between adjacent cells. The oligomerization of six Cx subunits forms a hemichannel called connexon and assembling of two connexons build up a complete GJ [11]. Mutations in the related genes of some members of the Cx gene family such as Cx26, Cx29, Cx30, Cx31, Cx32, Cx30.3, and Cx43 have been shown to lead to HL [12–14] and can be inherited either in monogenic or digenic mode. Furthermore, Cxs have a great potential to form different combinations of heteromeric and heterotypic GJ assemblies [15]. In Iran 18.29% of ARNSHLs are caused by *GJB2* mutations, 22.8% of which, that is 4.17% of total ARNSHL cases, would carry only one mutant allele in *GJB2* gene [16]. Digenic inheritance hypothesis in *GJB2* heterozygous cases has previously been proved for *GJB6* and *GJB3* alterations to comprise the second mutant allele [17–21]. A few studies have assessed *GJB4* [22–24], *GJA1* [23], and *GJC3* [23,25] genes in

**Abbreviations:** Cx, connexin; GJ, gap junction; HL, hearing loss; ARNSHL, autosomal recessive non-syndromic hearing loss.

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**Fig. 1.** Sequencing electropherogram of detected variations. (A)–(D) *GJB4* PCR products, below: WT allele. Above: heterozygous alleles (indicated by arrows): (A) 451C>A, (B) 219C>T, (C) 507C>G, (D) 542C>T. (E) and (F) *GJA1* PCR product, below: WT allele. Above: heterozygous alleles: (E) 758C>T, (F) 717G>A, (G) *GJB4* PCR product, above: WT allele, middle and below: 155\_158delTCTG heterozygous allele sequenced in two directions. (H) *GJA1* PCR product, above: WT allele, middle and below: c.3\*dupA heterozygous allele sequenced in two directions.

ARNSHL cases, though their chief aim was not to appraise the digenic hypothesis.

In the present investigation, we have investigated the contribution of *GJB4*, *GJA1*, and *GJC3* in *GJB2*-related ARNSHL patients with only one mutant allele.

2. Methods

*Subjects and control samples.* A total of 34 deaf subjects and 400 normal hearing individuals were included in this study. All deaf

**Table 1**  
Genotypes and frequencies of study subjects.

Heterozygous <i>GJB2</i> variants	Frequency	Percent
35delG/WT	30/34	88.23
E47X/WT	1/34	2.94
235delC/WT	1/34	2.94
W24X/WT	1/34	2.94
R184P/Wt	1/34	2.94
Total	34	100

**Table 2**PCR conditions for amplification of *GJB4*, *GJA1*, *GJC3*, and *GJB2* coding exons.

Gene	Primer directions	5'–3' primer sequences	Product size (bp)	Region	Annealing for PCR (°C)
<i>GJB4</i>	Forward	F1B4: TCAATCGCACCAGCATTAAG F2B4: GCTTCCTCTATATCTTCGAC <sup>a</sup>	964 407	Exon 2	Touch down 61 to 56
	Reverse	R1B4: GGGGGACCTGTTGATCTTATC			
<i>GJA1</i>	Forward	F1A1: TTTCGAATCTGTGATCCTTGA F2A1: CTGATGACCTGGAGATCCAG <sup>a</sup>	1281 93	Exon 2	54
	Reverse	R1A1: CCTGGTGCACCTTCTACAGC			
<i>GJC3</i>	Forward	F1C3: GCTCCCTCTGAAGGACAGTG	896	Exon 1	Touch down 65 to 58
	Reverse	R1C3: GGGAGGAGATCATCAGGACA			
	Forward	F2C3: TGGGTACGCACTGTGAAAAA	190	Exon 2	60
	Reverse	R1C3: AGCTCCTCTTGGACAGGAT			
<i>GJB2</i>	Forward	F1B2: CTCCTGTCTCTGCTCTAGCT	809	Exon 2	Touch down 62 to 58
	Reverse	R1B2: CTCATCCCTCTCATGCTGTC			

<sup>a</sup> Semi-nested PCR primers. Mismatch nucleotides are given in italics.**Table 3**

Full report of detected variations.

Variant genotype <sup>a</sup>	Amino acid change <sup>a</sup>	Primary <i>GJB2</i> genotype	Protein domain	Predicted effect	Patients freq. (%)	Control Freq.	SNP <sup>c</sup>	RFLP enzyme	Ref.
<i>GJB4</i>									
c.451C>A/Wt	(Arg151Ser)	35delG/Wt	E2	Missense	1/34 (2.94)	1/100	rs78499418	BsiEI	This study
c.219C>T/Wt	(p.=)	35delG/Wt	M2	Silent	1/34 (2.94)	–	rs143547547	–	This study
c.507C>G/Wt	(p.Cys169Trp)	35delG/Wt	E2	Missense	1/34 (2.94)	7/100	rs79193415	StyI	[22]
c.155_158delTCTG/Wt	(p.Ala52ValfsX55)	35delG/Wt	E2	Frameshift	1/34 (2.94)	5/100	NR	Tth1111	[22]
c.542C>T/Wt	(p.Thr181Met)	R184P/Wt	E2	Missense	1/34 (2.94)	0/100	rs142410428	NcoI	This study
<i>GJA1</i>									
c.758C>T/Wt	(p.Ala253Val)	35delG/Wt	C	Missense	1/34 (2.94)	1/100	rs17653265	HhaI	[32,33]
c.717G>A/Wt	(p.=)	35delG/Wt	C	Silent	1/34 (2.94)	–	rs57946868	–	This study
c.3*dupA/Wt	(p.?) <sup>b</sup>	35delG/Wt	C	Unknown	2/34 (5.88)	2/100, 4/100	rs67678923	BsII	This study

M, transmembrane domains; E, extracellular loop domains; C, C-terminal domain; NR, not reported.

<sup>a</sup> HGVS (Human Genome Variation Society) nomenclature.<sup>b</sup> Protein has not been analyzed, an effect may be expected but too complex to predict.<sup>c</sup> Reported in database of Single Nucleotide Polymorphisms (dbSNP).

subjects were ARNSHL cases with only one mutant *GJB2* allele (Table 1) identified in 890 affected families with mild to profound sensorineural HL in our previous study [16]. We collected the control samples from 4 provinces (100 for each region) based on the geographic region from where patients with new allelic variants were detected. Informed consent was obtained from all deaf subjects and the controls. The study protocol was approved by the Ethics Committee of Shahrekord University of Medical Science, Iran.

**Molecular analysis.** Genomic DNA was extracted from peripheral blood of the deaf subjects and control individuals, using a standard phenol–chloroform procedure. Polymerase chain reaction (PCR) reactions were performed to amplify the entire coding sequence of *GJB4* (RefSeq ID: NM\_153212), *GJA1* (RefSeq ID: NM\_000165), *GJC3* (RefSeq ID: NM\_181538), and *GJB2* (RefSeq ID: NM\_004004), plus sequences of about 50 bp up/down stream. Except *GJB2* primer sequences which we reported in our previous study [26], other primers were designed using Primer3 (v. 0.4.0) web-based software<sup>1</sup> [27] (Table 2) and were blasted (NCBI/Primer-BLAST<sup>2</sup>) in order to check their specificity. The Quality of PCR products were verified on poly-acrylamide gel electrophoresis (PAGE). DNA sequencing of the PCR-amplified products was carried out bi-directionally on an ABI 3130 automated sequencer (Applied Biosystems, MacroGen, South Korea) using the same primers.

Sequencing data were then analyzed comparing with published (UCSC Genome Browser<sup>3</sup>) sequences. The found variants were

investigated in 100 geographically and ethnically matched control samples by subjecting the related PCR product to the procedure of restriction fragment length polymorphism (RFLP). All products with positive RFLP results were sequenced. The *GJB2* gene was sequenced in all control individuals with variants in any of the three genes. Conservation scores of the amino acids, which were predicted to change, were acquired from the ConSurf Server (the online server for the identification of functional regions in proteins<sup>4</sup>) [28].

### 3. Results and discussion

Altogether, eight different heterozygous allelic variants were identified in the three genes in 9 of 34 (26.47%) deaf subjects studied (Table 3) (Fig. 1). Any variants found in the control individuals for *GJB4* or *GJA1* using RFLP, were confirmed by DNA sequencing. The *GJB2* gene sequence was normal in all the control individuals carrying variants in *GJB4* or *GJA1*.

Analyzing *GJB4* gene, we detected five different variants in 5/34 (14.70%) of the patients and 13/200 (6.5%) of the controls. Out of the 5 patients, 4 were heterozygote for 35delG *GJB2* mutation and one carried R184P. The first variant c.451C>A (Arg151Ser) was found in a patient from Golestan province in north of Iran. A semi-nested PCR was performed using F2B4 primer with a mismatch nucleotide (Table 2) to produce a restriction site for BsiEI enzyme. We found one heterozygote in 100 controls from the corresponding province. This allelic variant results in a substitution of arginine to serine at codon 151 of Cx30.3 within the second extracellular loop domain affecting a residue with a low conservation score of 2

<sup>1</sup> <http://frodo.wi.mit.edu/primer3/> (last accessed 4.10.12).<sup>2</sup> <http://www.ncbi.nlm.nih.gov/tools/primer-blast/> (last accessed 2.28.12).<sup>3</sup> <http://genome.ucsc.edu/cgi-bin/hgGateway/> Created by the Genome Bioinformatics Group of UC Santa Cruz, ©The Regents of the University of California (last accessed 2.23.2012).<sup>4</sup> <http://consurf.tau.ac.il/> (last accessed 1.15.12).

**Table 4**

Conservation status of missense variations.

Gene	Protein	Position	Amino acid	Conservation score <sup>a</sup> (Scale <sup>b</sup> )	MSA data <sup>c</sup>	Residue variety
<i>GJB4</i>	Cx30.3	151	R	0.718 (2)	150/150	F,A,S,T,N,K,Y,V,H,Q,M,C,I,R,L
		73	H	−0.812 (9)	150/150	H,Q,N,L,Y
		169	C	−0.943 (9)	150/150	A,C
		181	T	−0.847 (9)	150/150	A,S,M,T
<i>GJA1</i>	Cx43	253	A	2.005 (1)	78/150	F,S,T,N,K,Y,E,V,Q,C,L,A,P,H,D,R,I,G
		239	R	0.665 (2)	142/150	A,S,T,N,K,Y,E,V,H,Q,C,I,R,G

<sup>a</sup> The normalized conservation scores.<sup>b</sup> Scale representing the conservation scores (9 – conserved, 1 – variable).<sup>c</sup> The number of aligned sequences having an amino acid (non-gapped) from the overall number of sequences at each position.

(Table 4). This domain is crucial for docking of GJ hemichannels and is responsible for the compatibility between different connexin proteins to form heterotypic functional GJ channels [29]. However, considering that the altered arginine was a non-conserved amino acid with possibly low functional property in the protein, and observing the variation among healthy control samples, there would be a low possibility of its contribution to the disease.

Another variation was c.219C>T, found in one of the deaf subjects from Gilan in north of Iran, accompanying *GJB2* 35delG allele. It does not change the highly conserved histidine codon in position 73 in the second transmembrane domain and is considered a silent (synonymous) variation. Although the integrity of this domain is essential for the accurate transport of connexin into plasma membrane, it seemed fairly improbable for a silent variation to play a role in disease pathogenesis. No control samples were screened for that. More studies are necessary to specify any possible role of this variation on protein levels or conformation [30].

The variant c.507C>G (p.Cys169Trp), found in a patient from Azerbaijan Sharqi, Northwest Iran, was in double heterozygosity with *GJB2* 35delG. This patient had two other siblings with HL. The altered cysteine is a residue with a high conservation score of 9 (Table 4) substituted by tryptophan at codon 169 of Cx30.3 in the second extra cellular domain. Therefore, this alteration may result in incompatible forming of heterotypic functional channels. As the variation generated an additional restriction site for *StyI*, using the restriction enzyme, we detected seven heterozygotes carrying this variant in 100 control samples from the same region. As the *GJB2* sequence of all the healthy control individuals was normal, it can be regarded as the evidence of possible digenic effect between *GJB4* and the mutant *GJB2*. Cys169Trp has previously been suggested to contribute to HL in two studies based on the fact that its observed frequencies in patients were significantly higher than those of the controls [22,23].

In one patient from Azerbaijan Sharqi, with *GJB2* 35delG mutation, a 4 bp deletion (c.155\_158delTCTG) was detected in *GJB4*. The patient was the only affected person in his family. This allelic variation causes a frameshift generating a new stop codon at position 55 (counting starts with the changed amino acid). This deletion eliminated a restriction site of *Tth111I* enzyme. Among 100 control samples from Azerbaijan Sharqi, five had this variant in heterozygote form. López-Bigas et al. [22] proved that this variation is not a cause of HL since they found no significant difference between patient and control groups in this variant. In view of digenic hypothesis, this protein with the premature stop codon that would be exposed to degradation in the cell, would have minimal effect on the HL pathogenesis [31].

The c.542C>T variant was detected to be double heterozygous with *GJB2* R184P mutation in one patient from Azerbaijan Sharqi. He had another affected brother. The variation substituted a

tryptophan, with the high conservation score of 9 (Table 4), to methionine in second extra cellular loop domain of Cx30.3. While the tryptophan residue is present in the sequences from a diverse set of taxa including fish, Primata and rodent, M residue is only seen in *Xenopus* sp. based on the multiple sequence alignment involving 150 sequences from different sources. Thus, it is very probable to negatively affect the hemi-channel docking and the co-assembly of *GJB4* with different connexins in a heterotypic channel. This variant introduced one restriction site for *NcoI*. We did not detect this allelic variation in any of 100 control individuals from Azerbaijan Sharqi. Therefore Among our detected *GJB4* variations T181M is the most likely variant to contribute to HL in digenic mode together with the *GJB2* mutation R184P.

Analysis of *GJA1* gene showed 3 variants in 4/34 (11.76%) patients and 7/200 (3.5%) of the controls. They all carried 35delG as the primary *GJB2* mutation. One patient from Khuzestan, in southwest Iran, was found to carry c.758C>T in *GJA1* along with a 35delG mutation in *GJB2*. The variant was seen in 1/100 of the controls from Khuzestan province. The variant results in a substitution of an alanine with a low conservation score of 1 (Table 4), to valine in unusually long cytoplasmic C-terminal domain of Cx43. This domain of the protein is the only part of it that includes functional phosphorylation sites for kinases. In the presence of this variation, one of the restriction sites for *HhaI* was abolished. The variation has been previously reported to contribute to glaucoma [32] and oculodentodigital dysplasia [33]. Taking into account that the altered alanine is a non-conserved amino acid, it is less likely to have a contributory effect on HL pathogenesis in this case.

The c.717G>A was detected in one patient from Khuzestan, in double heterozygosity with *GJB2* 35delG mutation. It caused no change in arginine residue in cytoplasmic C-terminal domain of Cx43. This variation seems to have no causative effect on protein and/or disease pathogenesis.

A variation of c.3\*dupA, in which the third nucleotide downstream of the stop codon was duplicated, was found in one patient from Khorasan Razavi, northeast Iran, and in another case from Azerbaijan-Sharqi, both being in double heterozygosity with *GJB2* 35delG. A mismatch forward primer (F2A1) was designed (Table 2) to conduct a semi-nested PCR which generated a restrictions site of *BslI*. This variant was found in two of 100 control individuals from Khorasan Razavi and 4/100 of controls from Azerbaijan Sharqi. Since this variant is not located in the coding sequence (3' UTR), the possible outcome is currently unclear and warrants functional studies.

We detected no variations in the two exons of *GJC3* gene and all the 34 patients showed normal sequences. In the present study, we tried to clarify the possible contribution of digenic inheritance in ARNSHL in Iran and to identify probable suitable genes to be assessed in this issue. In this study, 26.47% of *GJB2* heterozygotes had a second variant in another Cx gene, actually being double

heterozygote. Notably, none of the controls were in double heterozygosity status for the studied genes and *GJB2*. Thus, a given variant in one of the studied Cx genes might be pathogenic when accompanied by another variant in *GJB2* in a digenic pattern. We propose that *GJB4* T181M variant and with lower possibility some other variations of *GJB4* and *GJA1*, but not possibly *GJC3*, can contribute to ARNSHL in a digenic pattern in *GJB2* heterozygous mutation carriers. Further functional and familial studies are required to definitely confirm the role of other variations of *GJB4* and *GJA1*, and to determine whether other members of the Connexin gene family play any role in the digenic hypothesis in HL.

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